Present Status of Handling Water Samples*

Comparison of Bacteriological Analyses Under Varying Temperature and Holding Conditions With Special Reference to the Direct Method

ELFREDA L. CALDWELL AND LELAND W. PARR, PH.D.
Director and Chief Bacteriologist, Field Research Laboratory, Alabama State Department of Health, Andalusia, Ala. †

ACCORDING to *Standard Methods of Water Analysis* of the American Public Health Association [1] the time required between collection of a sample for bacteriological analysis and the beginning of the analysis should not exceed 6 hours for impure water nor 12 hours for relatively pure waters; and during the period of storage the temperature should be kept between 6° and 10° C.; and any deviation from these limits should be so stated in making reports.

To determine to what extent and for what reasons deviations from this standard exist a questionnaire to each state and insular official [2] responsible for the water analysis of his particular area requested information on the use of ice in transporting and storing samples and the time elapsing between sampling and analysis. Fifty replies were received, only 2 insular authorities failing to respond.

Analysis of the replies indicates that the use of ice in the storage and transportation of water samples intended for bacteriological analysis is a rapidly declining practice. Fourteen states have given up the use of ice entirely, of which 5 are southern subject to fairly long seasons of summer heat, though none from the extreme South, and another after experimentation reports its intention to do so shortly. Including this state, only 11 state and insular laboratories reported the examination of iced samples only; 25 laboratories analyze both iced and un-iced samples, though in 6 of these the practice is tantamount to examination of un-iced samples. One or more of the following conditions govern the procedure in the other 19 laboratories:

a. Iced when collected by the Board of Health; otherwise un-iced
b. Iced in hot weather
c. Municipal samples iced, private samples un-iced.
d. Iced when not examined promptly.

The reasons given for the increasing use of un-iced samples may be resolved into the following points:

a. With modern transportation facilities, because of their size some of the smaller

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states and, through the establishment of branch laboratories, several of the larger states, feel that laboratory service has been brought within a few hours of all parts of the state.

b. Climatic conditions in some northern states preclude the need for ice.

c. Icing is expensive, often not practicable in remote areas, and frequently not carried out properly.

d. Un-iced mail samples have been found economical and practicable.

e. Many states do not ice if the analysis is carried out within 24 to 30 hours after sampling, since they consider icing necessary chiefly to safeguard the total counts on plates, which they omit except in certain technical controls.

No laboratory gave information as to the temperature of samples, iced or un-iced, at the time of analysis although several remarked that the use of ice had not been entirely satisfactory.

It is not improbable that the work of Berry [3] has considerably influenced this tendency toward the use of un-iced samples. He concluded:

> The number of Colon group bacteria in samples of ground water did not change materially in the first 48 hours after collection regardless of whether the samples were kept at ordinary temperatures or packed in ice. Samples shipped without ice-packing will, therefore, yield dependable results and may be safely utilized where ice-packing is impracticable.

Not all the 50 laboratories answering the questionnaire gave information as to time elapsing between sampling and analysis, and many of the replies are open to misinterpretation. Of those making definite statements we interpreted that:

13 states begin analysis within 24 hours after collection

15 states within 48 hours

6 analyze samples sometimes 3 or more days old.

It is unquestionably true that despite modern transportation facilities, a considerable number of water samples are more than 2 days enroute and a large number are not examined within 24 hours.

In the study of possible contamination of water supplies from latrines bored or excavated into ground water, it was important that the recovery of the test organism should indicate the true character of the ground water being tested. To minimize uncertainties due to the shipment of water samples, we adopted the "direct" method as standard. Samples were inoculated into lactose broth (3, 10 c.c.; 2, 1 c.c.; and 2, 0.1 c.c. volumes) and plated in Endo (1 c.c.) on the field immediately after collection of the test samples and examinations were completed at the Field Research Laboratory. Because of the large volume of work involved it was not possible to incubate the cultures immediately on the field The time interval between inoculations and placing cultures in the incubator ranged from 15 minutes to 5 hours with an approximate average of 3 hours. Frequently atmospheric temperatures approximated 37° C. or were sufficiently high for growth. In any case direct inoculations served to fix the test volumes in culture before changes in water samples could occur.

Methods Compared—In view of the varying practices summarized in Part I, it seemed worthwhile to compare our findings by the "direct" method with analyses of samples collected at the same time but examined after being held for varying periods under temperature conditions representative of these procedures. Samples were, therefore, collected from the same source at the same time and treated as follows:

Direct (D), as indicated above

Iced (I), ice-packed when collected and examined within a few hours

Un-iced (U), held at atmospheric temperatures and examined simultaneously with the iced

Express-Iced (EI), ice-packed when collected and held for 24 to 30 hours before examination

Express-Un-iced (EU), held at atmospheric temperatures and examined simultaneously with the express-iced

There were 672 duplicate samples examined direct and iced only, and 316 by all 5 methods. Though occasionally iced samples were held for 7.5 hours, by far the greater proportion were examined within 4 to 5 hours. When atmospheric temperatures were high and the holding period brief, the final temperatures of the iced samples did not fall to 10° C., occasionally registering 13° to 15° C. The majority recorded less than 10° C. As frequently happens in the shipment of samples to central laboratories, in very hot weather the ice melted in 24 to 30 hours. The final temperatures of express iced samples were, therefore, higher at times than 10° C., in cold weather recorded a low of 4.5° C., and averaged 17.1° C. for quintuplicate examinations. Un-iced samples fluctuated from 15° to 36.2° C. The temperature of the ground water, varied from 16.5° to 25.0° C., averaging 19.9° C.

Source of Samples—We had the advantage of a more exact knowledge of our sources than is generally possible. The greater proportion of samples derived from pipe wells in our experimental fields afforded waters of varying quality: (1) good water, (2) yielding *B. aerogenes* (soil) from infected pumps,[4] and (3) receiving contamination or having been contaminated from experimental latrines. Group 4 includes a few samples from outside sources representative of water of poor quality contaminated from the surface and handling only—*i.e.*, from shallow, unprotected dug and driven wells and surface waters.

Method of Bacteriological Examinations—In addition to *Standard Methods* for confirmed tests, the methyl red, indol and citrate tests were routinely employed to differentiate the colon-aerogenes group (hereinafter designated C-A) into 3 standard types—*B. coli*, *B. aerogenes* and Intermediates.[5] In a very careful search for *B. coli*, streakings were made from 2 dilutions at the end both of 24 and 48 hours, if gas production warranted. In general a minimum of 4 to 8 colonies were picked according to gas production in varying dilutions. The presence of anaerobes of the *Cl. Welchii* type was confirmed. Pseudomonas complications were controlled by successive re-platings. In the examination of Endo pour plates, colonies were grouped into (a) "C-A reds" and (b) "Others"—tiny whites predominating among the latter. Since 1 c.c. was routinely employed to obtain an accurate picture of C-A reds, when other colonies exceeded 1,000 in number they were not further estimated but recorded as > 1,000.

For simplicity we shall compare our

TABULATION A

Collections	#Samples	Method	Time in Hours		Temp. Water °C.		Maximum Air Temp.	
			Range	Ave.	Range	Ave.	Range	Ave.
185	672	D	0	0	16.0—19.5	16.7	11.0—28.0	22.0
		I	2.0— 7.5	4.5	2.0—13.0	8.6		
64	316	D	0	0	16.5—25.0	19.9	19.0—45.6	28.7
		I	1.0— 7.5	4.5	4.0—15.0	10.0		
		U	1.0— 7.5	4.5	17.0—34.9	25.7		
		EI	24.0—30.0	27.8	4.5—25.0	17.1		
		EU	24.0—30.0	27.8	15.0—35.8	26.9		

findings in terms (1) of the recovery of the C-A group as a whole, and (2) of its component member, *B. coli.*

THE FINDINGS

Direct and Iced Only—Since it had not been demonstrated that the direct method was feasible or would offer any advantages over iced samples inoculated within a few hours and incubated immediately, 672 duplicate direct and iced samples were analyzed. The very low recovery of lactose fermenters (1.0 per cent) and absence of C-A reds on pour plate from 604 duplicate samples of good water indicated that direct inoculations were practical. Contamination of cultures or plates in the field was not apparent. The recovery in 68 samples from wells receiving contamination suggested also that direct examinations were advantageous. The iced samples afforded 68 per cent of the direct recovery of the C-A group as a whole, and though a comparable number (95.5 per cent) yielded typical *B. coli* due to the gross contamination of receiving wells the C-A count on pour plate approximated only one-tenth.

Quintuplicate Comparison—Table I gives the numerical data for bacteriological analyses of samples in the different groups by the direct method, and compares the findings by the other methods in terms of the direct (percentage).

It is clear that in all groups the time interval before examination is of prime importance in the recovery of organisms of the colon-aerogenes group from water samples. Icing, though of secondary value, in general contributes definitely to recovery.

A. The direct method demonstrates its superiority by slightly higher gas production, significantly greater recovery of the C-A group as a whole, and notably higher yield of *B. coli,* evidenced not only by the greater

TABLE I

COMPARISON OF RESULTS OF BACTERIOLOGICAL ANALYSIS OF QUINTUPLICATE SAMPLES UNDER THE CONDITIONS INDICATED

Group	Wells	Collections	No. Sam.	Method×	No. Gas pos.	No. C-A pos.	No. B.c. pos.	Pour Plate Data			
								No. pos. C-A Reds	Total Reds	No. pos. Others	Total Others†
1	6	7	54	D	0	0	0	0	0	31	1,678
				Only occasional gas, no recovery of C-A group by any method.							
2	4	7	55	D	26	25	0	10	132	40	3,302
				I	61.5	60.0	..	50.0	8.3	102.0	140.0
				U	73.1	68.0	..	60.0	10.0	130.0	204.0
				EI	57.7	52.0	..	20.0	6.0	100.0	122.0
				EU	61.5	56.0	..	40.0	14.4	132.0	396.0 (2)
3	17	31	184	D	162	124	93	84	8,960	182	44,670 (16)
				I	92.6	80.6	59.1	63.1	20.0	98.9	58.9 (16)
				U	98.8	56.4	49.5	61.9	17.0	99.4	77.6 (18)
				EI	88.9	38.7	43.0	36.9	10.8	95.1	73.6 (17)
				EU	83.9	34.7	37.5	33.3‡	12.8‡	101.1	272.9 (92)
4	15	19	23	D	20	19	8	15	639	23	3,615 (1)
				I	95.0	94.7	87.5	100.0	70.6	100.0	205.7 (5)
				U	90.0	94.7	75.0	80.0	52.6	100.0	205.2 (5)
				EI	80.0	84.2	75.0	100.0	71.0	100.0	218.3 (3)
				EU	90.0	94.7	50.0	100.0	205.8	100.0	285.3 (5)
Total	32	64	316	D	208	168	101	109	9,731	276	53,265 (17)
				I	89.8	79.2	61.4	67.0	23.1	96.4	75.2 (21)
				U	94.2	62.5	51.2	64.2	19.2	107.2	92.5 (23)
				EI	85.6	45.8	45.5	44.0	14.6	96.4	86.7 (20)
				EU	82.7	44.6	38.6	50.0‡	44.8‡	112.3	288.8 (99)

× Numerical data for D; I, U, EI, EU expressed in terms of D (percentage).
† Number in ( ) indicates plates showing counts greater than 1,000.
‡ Discounting 29 counts from 124 plates in Group 3 not possible to indicate accurately.

percentage of positive samples but by the higher proportion of pour plates showing C-A colonies in greater numbers. There is progressively less recovery by other methods in the order: iced, un-iced, express iced and express un-iced.

B. Recovery from iced samples examined within a few hours is definitely and significantly greater than from the remaining 3 methods under the conditions of fecal or surface contamination usually encountered in the examination of water supplies. In handling samples yielding certain soil *B. aerogenes* only, icing apparently accelerates the death rate.

C. Though the difference in recovery of C-A organisms as a group from iced and un-iced samples held for 24 or more hours is slight, possibly because of the susceptibility of certain *B. aerogenes* to cold temperature, the yield of *B. coli* in favor of the express iced probably is significant.

Of special interest are the findings of 57 samples chosen from Group 3 from wells which, with 1 important exception mentioned later, had been consistently showing fecal *B. coli* derived from latrine contamination over a considerable period of time. With the exception of 1 sample showing gas due to anaerobes, the remaining 56 yielded *B. coli* upon direct examination in all samples, approximately 70 per cent in 1.0 c.c. or smaller volumes. The results are tabulated in Table II.

In these samples the standard iced method offered little better than one-half the direct recovery of *B. coli,* the un-iced express less than one-fourth, while the un-iced and express iced yielded approximately 33.0 per cent. Pour plates from samples, iced or un-iced, yielded approximately one-fifth of the direct C-A count when inoculated within a few hours and less than one-tenth when samples were held from 24 to 30 hours. In the un-iced express samples the marked loss in red colonies, 4.0 per cent of the direct, is in contrast to the increase in "other" colonies which were more than twice as numerous.

Among these wells one (X) is of particular interest. In contrast to the others this had been giving good water for approximately 2 months while pumping 40 gallons. In withdrawing 240 gallons fecal *B. coli* were pulled into the well, recoverable in all samples tested, yielding 1 to 6 *B. coli* per c.c. in 4 out of the 5 samples by the direct method. In this significant situation typifying a well affording consistently good water which under unusual conditions yielded dangerous contamination, the other methods contributed samples positive for *B. coli,* in 10 c.c. volumes only, as follows: iced 3; iced express 2; un-iced and un-iced express 1 each. A second well of this group showing gross contamination with *B. coli* in all 5 samples by the direct method, yielded *B. coli* also by the other methods, but in significantly less numbers evidenced by C-A colonies on pour plates: (D) 583; (I) 101; (U) 27; (EI) 37; and (EU) 26.

TABLE II

COMPARISON OF BACTERIOLOGICAL ANALYSES OF SAMPLES FROM WELLS ALL RECEIVING FECAL CONTAMINATION

Wells	Collections	No. Sam.	Method×	No. Gas pos.	No. C-A pos.	No. B.c. pos.	Pour Plate Data			
							No. pos. C-A Reds	Total Reds	No. pos. Others	Total Others*
12	18	57	D	57	56	56	39	827	56	20,696 (13)
			I	100.0	71.4	55.4	48.7	20.9	101.8	109.8 (16)
			U	100.0	44.6	35.7	41.0	23.0	101.8	108.6 (16)
			EI	100.0	33.9	32.1	25.6	6.1	101.8	129.0 (14)
			EU	100.0	32.1	23.2	20.5	3.9	101.8	206.7 (37)

* See Table I.

DISCUSSION

These results emphasize the points stressed by *Standard Methods of Water Analysis* that " because of the rapid and extensive changes which may take place in the bacterial flora of bottled waters when stored even at temperatures as low as 10° C., it is urged as of importance, that all samples be examined as promptly as possible after collection." Our series serves to emphasize anew the probable process of loss of bacteria significant of dangerous contamination—a rapid death rate of fecal organisms, particularly *B. coli,* and an increase in associated organisms. The particular wells mentioned support the general findings that in the light of results from direct inoculations the death rate of fecal *B. coli* in bottled waters is very rapid even within a few hours. Our results indicate that in bacteriological analyses of water receiving undoubted fecal and perhaps dangerous contamination the loss of recovery even with the iced samples examined within a few hours approximates 40 to 50 per cent, and there is progressively greater loss as the time of holding increases whether samples are iced or un-iced. It should further be stressed that in the examination of our samples the procedures involved a more thorough search for fecal organisms than usual in public health laboratories, and it follows that our findings probably show greater recovery than would obtain in the analyses of the same samples under routine conditions. Unless samples are inoculated directly, our results indicate the need of icing when waters are receiving significant fecal contamination and suggest that the increasing tendency to send samples un-iced is open to question. Though practical circumstances may make examination of un-iced samples necessary or even advantageous in handling certain waters, it should be emphasized that the practice derives sanction from expediency and does not conform in general to the conditions best for analysis.

CONCLUSIONS

We have found direct inoculation feasible. There is no question of the marked superiority of this method under any and all circumstances. We believe that with samples collected by state employees, particularly from water supplies of small villages and towns without laboratory facilities, in areas where bus transportation is available to carry cultures and plates to a central or branch laboratory for incubation within a few hours, direct inoculations are practicable. In water plants serving municipalities having laboratories, direct inoculations at the various stations could readily be adopted.

SUMMARY

An analysis of 50 replies received from state and insular laboratories indicates (a) that in most cases the procedure prescribed in *Standard Methods of Water Analysis* with respect to the storage and time of examination of water samples is not observed; (b) that a large number of water samples are not examined within 24 hours and a considerable number after 48 hours; (c) that ice-packing of water samples is rapidly declining. Comparison of recovery of colon group bacteria from iced and un-iced samples examined at varying periods indicates a very rapid death rate of *B. coli* in bottled samples of contaminated waters, which is progressively greater as the period of holding increases. Icing, though of secondary importance, offers a distinct advantage. Direct inoculations were found practicable and showed marked superiority over any other method. The feasibility of the direct method for municipal water supplies is suggested.

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